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Identification of 4-Demethylsterols from Suspension Cultured Cells of *Marchantia polymorpha* L.

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Five kinds of 4-demethylsterol were isolated from suspension cultured cells of a liverworth, *Marchantia polymorpha*. Four 4-demethylsterols among them were analyzed by a capillary gas chromatography-mass spectrometry and 500 MHz ^{1}H-NMR, and characterized to be avenasterol, 24-methylene-cholesterol, stigmasterol, and campesterol. And the fifth 4-demethylsterol was characterized to be sitosterol by a capillary gas chromatography-mass spectrometry. The concentration of the 4-demethylsterols in the cell decreased in order of avenasterol>stigmasterol>24-methylene-cholesterol>campesterol>sitosterol. When carbon skeleton and the oxidation state was compared with those of brassinosteroids, these 4-demethylsterols may be potent biosynthetic precusors of brassinosteroids in the cells.

Keywords : *Marchantia polymorpha*, suspension cultured cell, 4-demethylsterols, structure determination, precusors of brassinosteroids

Since a novel plant growth hormone, brassinolide was identified from rape (*Brassica napus* L.) pollen (Grove *et al.*, 1979), the presence of over sixty members of brassinolide related compounds (brassinosteroids) has been verified from various plant sources (Kim, 1991).

All the naturally-occurring brassinosteroids are known to be derivatives of 5α-cholestane. Diverse structural variations thus come from the kind and orientation of functionalities on the skeleton. Considering the structural characteristics with regard to biogenesis, brassinsteroids may be biosynthesized by further oxidation and hydroxylation from 4-demethylsterols which carry the very similar carbon skeleton to that of brassinosteroids and are contained as relatively large-amount components in plant tissuses (Yokota *et al.*, 1987). However, any experimental evidences for the biosynthetic pathway of brassinosteroids from 4-demethylsterols are not established yet.

In order to demonstrate the biosynthethic pathway of brassinosteroids from 4-demethylsterols, we selected suspension cultured cells of a liverworth, *Marchantia polymorpha* which showed relatively a strong brassinosteroids-activity as a test-plant material. And we firstly tried to identify endogenous 4-demethylsterols in the cells because the structural characteristics of the identified 4-demethylsterols, accompanied with those of brassinosteroids which are going to be identified now, give clues for feeding experiments for demonstrating the biosynthetic pathway of brassinosteroids. Here we report the isolation and the identification of 4-demethylsterols from the cell. In addition, some structural characteristics of 4-demethylsterols as potent biosynthetic precusors of brassinosteroids in the cell are discussed.

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MATERIALS AND METHODS

Plant materials

Suspension cultured cells of a liverworth, *Marchantia polymorpha* developed by Ohta (Ohta *et al.*, 1977) and maintained in Korea Research Institute of Bioscience and Biotechnology were used in this study. The cells were grown in a specially deviced two-layer Erlenmeyer flask (Husemann and Barz, 1977). In the upper-layer flask, approximately 1 g of cells of *M. polymorpha* were added in a mixture medium (60 mL) of M51 macronutrient plus vitamines (Furner *et al.*, 1978) and B5 micronutrient (Gamborg *et al.*, 1968) containing 1 g/L casamino acid, 0.2 g/L glutamine and 1 mg/L 2,4-D. And 50 mL of 2 M carbonate solution containing a mixture of 0.4 M K_2CO_3 and 1.6 M $KHCO_3$ to increase CO_2 concentration was added in the lower-layer flask. The suspension cells were grown for 10 days at 25℃ in light (5000 Lux) shaking (100 rpm) incubator.

Isolation of 4-demethylsterols

Extraction of phytosterols

The suspension cultured cells of *M. polymorpha* (500 g) were homogenized and extracted with 95% methanol (500 mL) three times. The extracts were reduced to dryness in *vacuo* and solvent-partitioned between H_2O and chloroform (500 mL) three times. Then the dried residues of chloroform-soluble fraction (Fr) were solvent-partitioned between *n*-hexane and 80% methanol (500 mL) three times. Finally *n*-hexane-soluble Fr (1 kg) containing phytosterols was obtained.

Saponification of phytosterols

The dried residues of *n*-hexane-soluble Fr were saponified with 80% ethanol containing 5% KOH at 70℃ for 90 min. Then the unsaponified lipids (900 mg) were extracted with *n*-hexane (200·mL) three times.

Silica gel column chromatography

The unsaponified lipids were charged on a column (2.2×220 mm) of SiO_2 (30 g) and eluted with a mixture (1 : 1) of *n*-hexane and dichloromethane. The obtained Frs were divided into four Frs namely non-lipid Fr, 4,4-dimethylsterols Fr, 4-methylsterols Fr and 4-demethylsterols Fr based on the movement on TLC (developing solvent: ethanol free chloroform) using a F254 preparative silica plate (Merck Co.).

Acetylation of 4-demethylsterols

4-demethylsterols Fr (ca 400 mg) was dissolved in acetic anhydride (1 mL) and pyridine (2 mL) and incubated at room temp. for overnight. Then the product was adjusted to pH 7 with HCl solution (pH 3) and extracted with *n*-hexane (200 mL) three times. After evaporation of *n*-hexane-soluble Fr, acethylated 4-demethylsterols (420 mg) was collected.

$AgNO_3/SiO_2$ column chromatography

$AgNO_3$ (5 g) was dissolved in acetonitrile (20 mL) and mixed with SiO_2 (20 g). The mixture was dried *in vacuo* and baked in heating oven at 110℃ for 1 hr. The 4-demethylsteryl acetate was charged on the $AgNO_3/SiO_2$ column and eluted with mixtures of *n*-hexane-benzene (200 mL of 9 : 1, 200 mL of 4 : 1 and 400 mL of 7 : 3). The obtained Frs were examined by $AgNO_3/SiO_2$ TLC using ethanol free chloroform as a developing solvent.

Reversed phase HPLC

The active Frs obtained from $AgNO_3/SiO_2$ chromatography were purified by a μ-bondapak C_{18} reversed phase HPLC column using methanol-hexane (97 : 3) as a mobile phase. The flow rate was 6 mL/min. and Frs were collected in every min. Finally three active Frs A (6.6 mg), B (2.7 mg) and C (23.2 mg) were collected (Table 1).

Strucure analysis

GC-MS

Table 1. Mobility of 4-demethylsteryl acetates identified from *M. polymorpha* cell on a reversed phase HPLC and a capillary GC

HPLC Fr	Compound	Retention time (min)	
		HPLC	GC
A	A	9.80	25.50
B	B	8.40	23.29
C	C	10.30	23.08
	D	10.30	23.45
	E	10.30	25.10

GC-MS analysis of 4-demethylsteryl acetate was accomplished by Hewlett Packard HP 5970B (ionization voltage, 70 eV) using a fused silica HP-1 capillary column (0.25 mm×25 m, 0.11 μm film thickness). The followings are the condition of GC: a splitless mode, 0.8 mL He/min, injection Temp: 270℃, Oven Temp: 150℃ for 5 min, thermal gradient 13℃/min to 280℃, and then 280℃.

^{1}H-NMR

Bruker 500 MHz FT-NMR was used for ^{1}H-NMR analysis. The sample was dissolved in $CDCl_3$ and tetramethylsilane (TMS) was used as an internal standard.

RESULTS

Several cultured cells were examined for brassinosteroids-activity by a rice lamina inclination bioassay (Wada *et al.*, 1981). Among the tested cells, suspension cultured cells of a liverworth, *Marchantia polymorpha* showed relatively a strong brassinosteroids-activity (Fig. 1). Thus suspension cell of *M. polymorpha* was selected as a plant material to examine biosynthetic pathway of brassinosteroids.

Suspension cultured cells of *M. polymorpha* (500 g) were extracted with aqueous methanol and solvent-partitioned as shown in Fig. 2. Lipids containing phytosterols and brassinosteroids were collected in hexane-soluble Fr and ethylacetate-soluble Fr, respectively.

After saponifying the hexane-soluble Fr, unsaponified lipids were divided into four Frs by silica gel column chromatography, namely non-polar lipids Fr, 4,4-dimethylsterols Fr, 4-methylsterols Fr and 4-demethylsterols Fr (Fig. 3). Then the 4-demethylsterols Fr was acetylated and further purified by an argentation column chromatography (SiO_2/$AgNO_3$). Finally Fr A, B, and C were separated by a reversed phase HPLC.

GC-MS analysis using a fused silica capillary column revealed that the reversed phase HPLC Fr A contains a 4-demethylsteryl acetate, designated as compound A (Table 1). The mass spectrum of com-

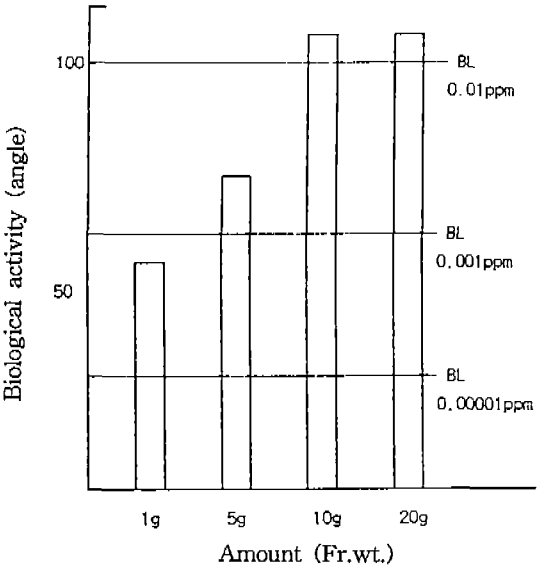

Fig. 1. Brassinosteroids activity in suspension culture cell of *M. polymorpha* by rice lamina inclination bioassay.

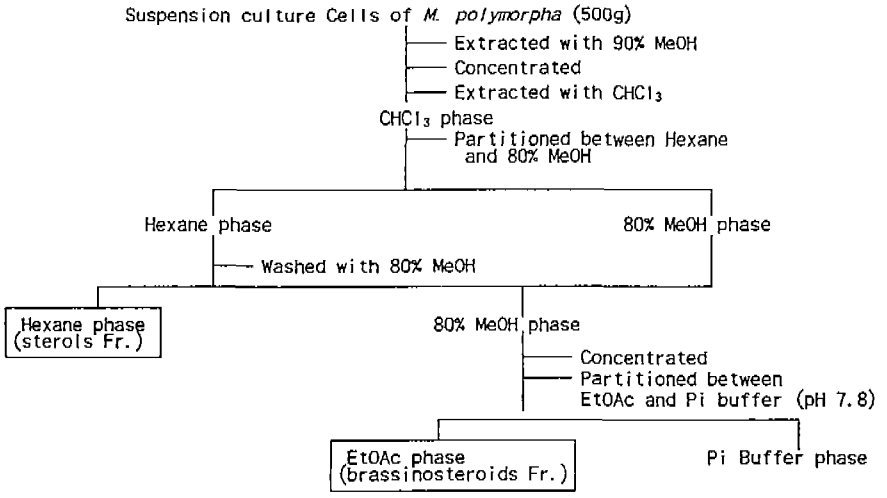

Fig. 2. Solvent partitioning procedure of phytosterols and brassinosteroids in suspension culture cell of *M. polymorpha*.

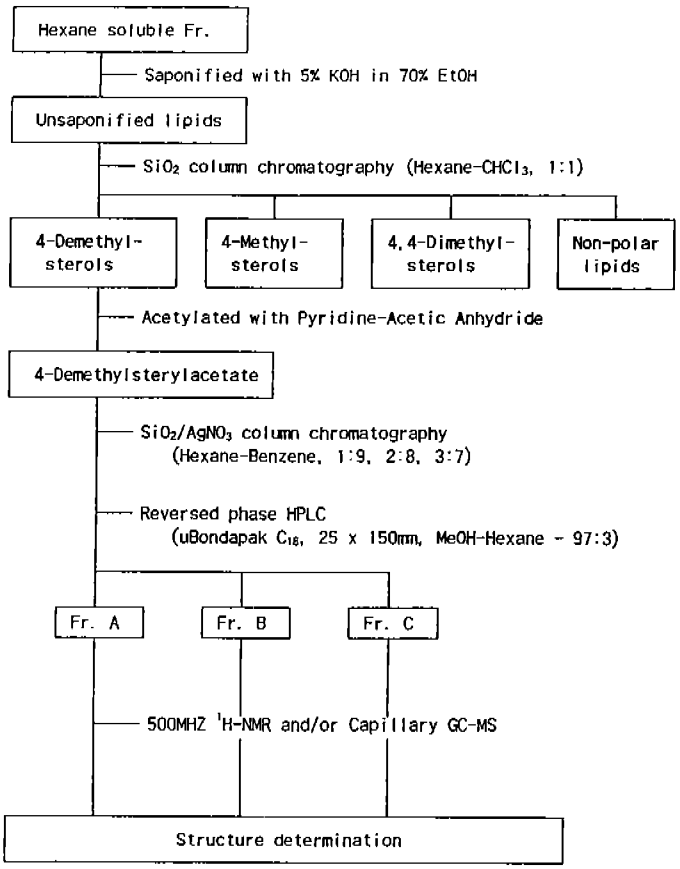

Fig. 3. Isolation and characterization methods of 4-demethylsterols in suspension culture cell of *M. polymorpha*.

Table 2. Characteristic fragment ions (*m/z* 150-500) in mass spectrum of 4-demethylsteryl acetates in *M. polymorpha* cell

Compound	Ion at *m/z* (relative intensity)						
A	394 (18),	296(100),	281(22),	253(11),	213(8),	211(6),	158(5)
B	380 (100),	296(53),	281(22),	253(28),	213(15),	211(9),	159(16)
C	382 (100),	367(25),	261(17),	255(18),	213(13),	159(11)	
D	394 (100),	351(12),	282(7),	255(37),	213(7),	211(4),	159(13)
E	396 (100),	354(5),	288(16),	255(20),	213(13),	159(11)	

pound A (Table 2) showed a strong ion at *m/z* 394, correponding to $[M-CH_3COOH]^+$, accompanied with fragmentation ions at *m/z* 296 (base peak, loss of acetic acid and fission of C22-C23 bond in side chain) indicating that it was an acetate of a C_{29}-sterol with two double bond, one of which was in side chain and the other was in the ring skeleton (Akihisa *et al.*, 1987). The presence of a significant ion at *m/z* 253 due to loss of acetic acid (AcOH) and side chain with 2H transfer suggested that the side chain double bond was located either at C24-C25 or C24-C28 position (Kim *et al.*, 1987). The proton signals were observed in the 500 MHz ^{1}H-NMR spectrum of compound A at δ 0.68 (s, H_3-18), 1.02 (s, H_3-19), 2.03 (s, H_3-3OAc), 4.60 (m, $W_{1/2}$=25 Hz, H-3) and 5.38 (br. d, J=10 Hz, H-6). Hence compound A are thought to possess a Δ5-3β-acetoxy-5 α-sterol skeleton. The side chain proton signals of compound A were observed at δ 0.95 (d, J=6.4 Hz, H_3-21), 0.98 (d, J=7.1 Hz, H_3-26 and H_3-27), 1.59 (d, J=5.8, H_3-29) and 5.11 (q, J=5.8, H-28), among which one olefinic quartet, together with the methyl doublet indicated that side chain double bond at C24 must be located at C24(28) as the terminal Z-

Table 3. 500 MHz ^{1}H NMR data (TMS internal standard) of 4-demethylsteryl acetates in *M. polymorpha* cell

Compound	H_3-18	H_3-19	H_3-21	H_3-26	H_3-27	H_3-28	H_2-28	H_3-29	H_3-3OAc	H-3	H-6	H-22 or H-23	H-28
A	0.68 s	1.02 s	0.95 d (6.4 Hz)	0.98 d (7.1 Hz)	0.98 d (7.1 Hz)			1.59 d (5.8 Hz)	2.03 s	4.60 m (25 Hz)	5.38 br.d (10 Hz)		5.11 q (5.8 Hz)
B	0.68 s	1.02 s	0.96 d (6.3 Hz)	1.02 d (6.7 Hz)	1.02 d (6.7 Hz)		4.66 d (1.4 Hz) 4.71 s		2.03 s	4.60 m (25 Hz)	5.38 br.d (10 Hz)		
C	0.68 s	1.02 s	0.91 d (6.3 Hz)	0.85 d (6.7 Hz)	0.80 d (6.6 Hz)	0.77 d (6.4 Hz)			2.03 s	4.60 m (25 Hz)	5.38 br.d (10 Hz)		
D	0.68 s	1.02 s	1.02 d (6.4 Hz)	0.85 d (7.2 Hz)	0.81 d (7.2 Hz)			1.02 t (6.3 Hz)	2.03 s	4.60 m (25 Hz)	5.38 br.d (10 Hz)	5.02 dd (8.8, 15 Hz) 5.16 dd (8.8, 15 Hz)	

s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad.

oriented ethylidene group (Baba *et al.*, 1983). Therefore Compound A was characterized to be avenasteryl acetate (Fig. 5A, isofucosteryl acetate, 24Z-ethylidene-cholesteryl acetate).

The reversed phase HPLC Fr B gave a single peak on total ion chromatogram (TIC) in GC-MS analysis, indicating that Fr B also contains a 4-demethylsteryl acetate, designated as compound B. In mass spectrum, a strong fragmentation ion at *m/z* 380 due to molecular ion minus acetate, $[M-AcOH]^+$ indicated the molecular weight of compound B is 440. Characteristic ions at *m/z* 296 (fission of C22-C23) and at *m/z* 253 (loss of side chain and acetate with 2H transfer) suggested the presence of two double bonds at C24-C25 or C24(28) in side chain and ring structure moiety. In 500 MHz ^{1}H-NMR, the two olefinic proton signals at C28 were observed at δ 4.66 (d, J=1.4 Hz, H-28) and 4.71 (s, H-28). The signal at δ 5.38 (br. d, J=10 Hz) was assigned as H-6. Also other proton signals were assigned as shown in Table 3. Thus compound B was characterized to be 24-methylene-cholesteryl acetate (Fig. 5B).

In GC-MS, reversed phase HPLC Fr C gave three peaks on TIC (Fig. 4). The compounds representing the peaks were referred to compound C, D and E based on their movement in the capillary column (Table 2). Mass spectrum of compoud C revealed a characteristic fragmentation ion, $[M-AcOH]^+$ at *m/z* 380 with a strong ion due to breakdown of side chain and acetic acid at *m/z* 255, which strongly suggests the presence of methyl group at C24 in saturated side chain structure. The signals at H_3-21, H_3-28, H_3-26 and H_3-27 in the ^{1}H-NMR were absorbed at δ 0.91 (d), 0.77 (d), 0.85 (d) and 0.80 (d), respectively. The signals derived from protons in ste-

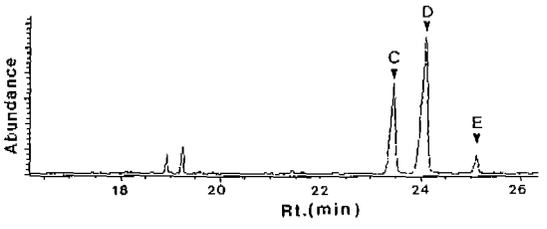

Fig. 4. Total ion chromatogram (TIC) of 4-demethylsteryl acetates in a reversed phase HPLC Fr C in GC-MS analysis. Condition; HP-1 capillary column (0.2 mm×25 m, 0.11 μm film thickness): injection Temp., 270℃; oven Temp., 150℃ for 5 min; gradient to 280℃ for 10 min; 280℃ for 15 min; carrier gas, He 0.8 mL/min; injection mode, splitless; ionization voltage, 70 eV.

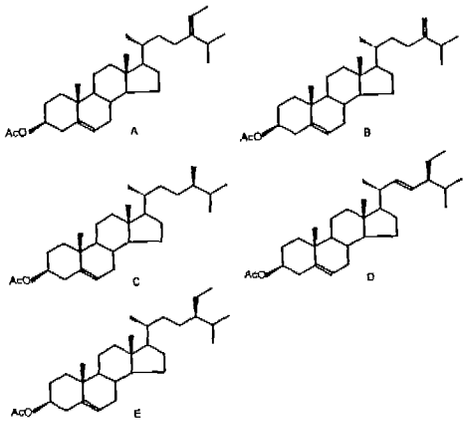

Fig. 5. Structure of 4-demethylsteryl acetates characterized in this paper. A, avenasteryl acetate; B, 24-methylene-cholesteryl acetate; C, campesteryl acetate; D, stigmasteryl acetate; E, sitosteryl acetate.

rol ring skeleton were identical those of avenasteryl acetate (compound A), Δ5-steryl acetate structure. Thus compound C was determined to be campesteryl (24*S*-methyl-cholesteryl) acetate (Fig. 5C).

The mass spectrum of compound D showed a characteristic ion of 4-methylsteryl acetate, $[M-AcOH]^+$ at *m/z* 394, which is the same fragmentation ion as that of compound A (avenasteryl acetate). However the characteristic ions at *m/z* 296 due to fission of C22-C23 bond and 253 due to loss of side chain and acetate with 2H transfer in mass spectrum of compound A were not found in that of compound D. Instead, strong fragmentation ions at *m/z* 352 (loss of isopropyl moiety and acetate) and 255 (loss of side chain and acetate) were observed, suggesting that the molecular formula of compound D is the same as that of compound A but the position of double bond at C24(28) in compound A is located at C22-C23 in compound D. The presence of C22-C 23 double bond in compound D was confirmed by olefinic proton double-doublets in the ^{1}H-NMR at δ 5.02 (H-22 or H-23) and 5.16 (H-22 or H-23). Also the triplet signal at δ 1.02 was assignable as methyl group at C29. Therefore, it is thought that compound D carry a *S*-oriented C24-ethyl moiety in the side chain. The signals of protons derived from sterol ring structure were assigned as follow: δ 0.69 (s, H_3-18), 1.02 (s, H_3-19), 2.03 (s, H_3-3OAc), 4.60 (m, $W_{1/2}$=25 Hz, H-3), 5.38 (br.d, J=10 Hz, H-6). Thus compound D was characterized to be stigmasteryl (24*S*-ethyl-22,23-dehydro-cholesteryl) acetate (Fig. 5D).

The minor 4-demethylsteryl acetate component, compound E was not isolated as a pure state. Thus it was characterized by a capillary GC-MS without ^{1}H-NMR. The retention time of compound E in GC-MS was 25.10′ which is identical to that of an authentic sitosteryl acetate (25.10′). The mass spectrum of compound E shown in Table 2 was also superimposed to that of the authentic sitosteryl acetate [*m/z* 396 (relative intensity, 100), 354 (6), 288(18), 255(22), 213 (13), 159(10)]. Therefore compound E was determined to be sitosteryl (24*S*-ethylcholesteryl) acetate (Fig. 5E).

DISCUSSION

From suspension cultured cells of *M. polymorpha*, we identified five kinds of 4-demethylsterols. The quantity of the 4-demethylsterols decreased in order of avenasterol>stigmasterol>24-mehtylene-cholesterol>campesterol>sitosterol. Since the side chain unsaturated 4-methylsterols in plant tissues are generally contained as larger-ammount components rather than the side chain saturated 4-demethylsterols and regarded as precusors of the side chain saturated 4-demethylsterols, the higher quantity of the unsaturated 4-demethylsterols (avenasterol, stigmasterol, 24-methylene-cholesterol) rather than the saturated 4-demethylsterols (campesterol, sitosterol) in the *M. polymorpha* cell is reasonable.

Yokota *et al.* (1987) have identified sitosterol and dihydrobrassicasterol as major 4-demethylsterols from a green alga, *H. reticulatum*. They have also identified 24*S*-ethylbrassinone and 24R-castasterone as major brassinosteroids from the same material, which carry the same side chain skeleton as that of the identified 4-demethylsterol. Kim *et al.*(1987b) identified castasterone and 25-mehtyldolichosterone as major brassinosteroids from an immature seed of *P. vulgaris*. The corresponding 4-demethylsterols with the same side chain skeleton, campesterol and 24-methylene-25-methylcholesterol were also isolated from the seed (Kim *et al.*, 1987a). Recently Schmidt *et al.* (1991) identified castasterone as a major brassinosteroids accompanied with campesterol from a seed of *Beta vugaris*. These findings strongly indicate that brassinosteroids are co-occurred with 4-demethylsterols carrying the same side chain structure as that of brassinosteroids in a plant tissue. Also these findings strongly suggest that brassinosteroids may be biologically synthesized by further oxidation and hydoxylation from 4-demethylsterols which have the same carbon skeleton as those of brassinosteroids and are contained in the plant tissues at higher concentration than brassinosteroids.

The identified 4-demethylsterols from *M. polymorpha*, avenasterol, 24-mehtylene-cholesterol, campesterol and sitosterol have the same side chain skeleton as those of brassinosteroids, homodolichosterone (or homodolicholide), dolichosterone (or dolicholide), castasterone (or brassinolide) and 24*S*-ethylbrassinone respectively. Thus it is highly possible that *M. polymorpha* cell also contains those brassinosteroids. If so, the identified 4-demethylsterols in this study could be used as starting compounds of fee-

ding experiments for examining the biosynthetic pathway of brassinosteroids from 4-demethylsterols.

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Marchantia polymorpha (우산이끼) 현탁배양 세포내의 4-methylsterol들의 동정

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적 요

하등 선태식물인 우산이끼(*Marchantia polymorpha* L.)의 현탁배양 세포로부터 5종류의 4-demethylsterol을 분리하였다. 이들 중 4종은 capillary GC-MS와 500 MHz ^{1}H-NMR를 이용하여 avenasterol, 24-methylene-cholesterol, campesterol 그리고 stigmasterol로 구조결정하였다. 나머지 1종은 capillary GC-MS만으로 분석하여 sitosterol로 구조결정하였다. 이들의 동세포내의 함량은 avenasterol>stigmasterol>24-methylene-cholesterol>campesterol>sitosterol의 순이었다. 이들 구조결정된 4-demethylsterol들의 탄소골격과 산화상태를 brassinosteroid의 그것들과 비교할 때, 이들 4-demethylsterol들은 brassinosteroid의 생합성 경로의 전구물질일 가능성이 크다고 사료된다.

주요어 : *Marchantia polymorpha*, 현탁배양 세포, 4-demethylsterol, 구조결정, brassinosteroid의 생합성 전구물질

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